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ORIGINAL ARTICLE

Isolation, identification and genetic organization of the ADI operon in *Enterococcus faecium* GR7

Baljinder Kaur · Rajinder Kaur

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Abstract L-Arginine is an indispensable amino acid, as it is required for normal growth of microbes, plants and animals (Szende et al., Cancer Cell Int 1:1475–1480, 2001). Arginine deiminase is the first enzyme of arginine deiminase (ADI) pathway, which catalyzes the conversion of arginine to citrulline and ammonia in an irreversible reaction. Lactic acid bacteria isolated from dairy products were investigated for their ability to hydrolyze arginine. Citrulline production in many LAB strains suggests that the arginine metabolism takes place via the arginine deiminase pathway. The highest arginine deiminase specific activity (0.27 IU/mg) was reported in isolate GR7, which was characterized morphologically, biochemically and by 16S rRNA gene sequencing as *Enterococcus faecium*. Genetic organization of the ADI operon in *E. faecium* GR7 was further studied using various molecular biology and computational techniques. Sequence analysis revealed that the genes involved in arginine catabolism are clustered together in an operon (3,906 bp) consisting of the genes *arcA* (arginine deiminase), *arcB* (ornithine transcarbamylase), and *arcC* (carbamate kinase), which are localized on the anti-sense strand of genomic DNA. Nucleotide sequence analysis revealed three open reading frames (ORFs) that were arranged contiguously and transcribed in the same direction, as an apparent operon. The genes followed the order *arcC*, *arcB*, *arcA*, which differs from that found in other microorganisms. The information obtained in this study provides the basis for testing the potential of arginine catabolism to control the emergence of arginine auxotrophic tumors.

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Keywords Arginine deiminase · *Enterococcus faecium* · Lactic acid bacteria · *arcA, arcB, arcC* · ADI reading frame

Introduction

Lactic acid bacteria (LAB) cannot biosynthesize functional cytochromes, and cannot get ATP from respiration. Energy metabolism of LAB is mainly based on sugar fermentation, arginine deamination, acid and amino acid decarboxylation, and on its proteolytic system (Pessione 2012). In a previous work, several strains of *Enterococcus faecium* have been isolated from dairy products, fermented foods, and plants such as raw milk, cream, cheese, butter, chicken, fermented milk products, nuka and dry sausages (Crow and Thomas 1982; Saavedra et al. 2003; Foulquié Moreno et al. 2006). Many strains are exploited to produce industrially important biomolecules, such as lactic acid, acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides and enzymes (Mittal et al. 1995; Caplice and Fitzgerald 1999).

Microorganisms catabolize arginine mainly by four pathways: the arginase pathway, arginine deiminase (ADI) pathway, arginine succinyltransferase pathway and arginine transaminase/oxidase/ dehydrogenase pathway. In LAB, arginine degradation occurs via the ADI pathway, which involves three enzymatic reactions. L-Arginine is hydrolyzed into L-citrulline and ammonia; this is an irreversible chemical reaction catalyzed by arginine deiminase. The resultant L-citrulline is decomposed into ornithine and carbamoyl phosphate by ornithine transcarbamylase (OTC), and this carbamoyl phosphate is further decomposed into ammonia and CO_2 by carbamate kinase (CK), as shown in Fig. 1. Hydrolysis of one mole of arginine yields two moles of ammonia and one mole each of ornithine, ATP and carbon dioxide, which is useful to compensate the acidity generated by sugar catabolism to lactic acid, acetic acid and formic acid,

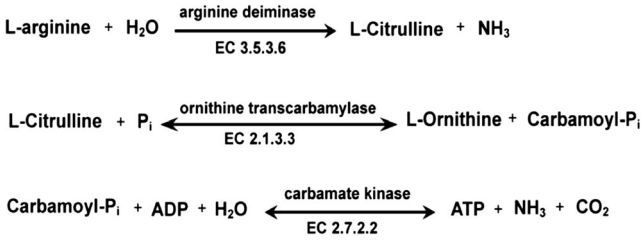

Fig. 1 L-Arginine catabolism via ADI pathway in LAB

in both homo- fermenting and hetero-fermenting conditions (De Angelis et al. 2002). In other words, the ADI pathway provides energy and protection against an acidic external pH. But, its relevance as an energy source or as a protective system against acidic environments varies among LAB.

The diversity in the gene organization of the ADI pathway and regulation of operons has been revealed by physiological and genetic studies (Zuniga et al. 2002). Most organisms studied so far possess ADI genes organized in single operon. The main cytoplasmic enzymes of the ADI pathway namely arginine deiminase, ornithine transcarbamylase and carbamate kinase are encoded by *arcA*, *arcB*, *arcC* genes respectively, are conserved in the *arc* operons of LAB. In addition to these genes, other genes may be present in *arc* operons of LAB, including *arcD* encoding arginine/ornithine antiporter, *arcT* encoding putative aminotransferase or transaminase, *arcR* encoding regulator. Arginine induces expression of ADI pathway enzymes and some carbohydrates such as glucose are reported to repress their synthesis, being controlled by catabolite repression (Crow and Thomas 1982).

This study was undertaken to examine the occurrence of ADI pathway enzymes in dairy LAB isolates, capable of catabolizing arginine. The correlation between the formation of ammonia and citrulline from arginine catabolism and the occurrence of ADI pathway enzymes was investigated using a simple procedure. Further, to gain deeper insight into the regulation of arginine catabolism, a 3,906 bp nucleotide sequence of *E. faecium* GR7 was sequenced and the genetic organization of various genes involved in the ADI pathway of *E. faecium* GR7 was characterized.

Materials and methods

Isolation, culture media and culture conditions

For the isolation of arginine-catabolizing LAB strains, various food products including milk and dairy products were collected from the Patiala (Punjab) region. Samples were mixed well with saline (0.85 % NaCl) and processed immediately for isolation of lactic acid bacteria. LAB were multiplied in enrichment media, i.e., modified MRS as described by De Angelis et al. (2002). The pure and healthy colonies of bacterial strains were further isolated by streaking on enrichment medium, i.e., MAM agar plates consisting of (g/l) tryptone 10.0; glucose 5.0; yeast extract 5.0; arginine 3.0; KH_2PO_4 0.5; $MgSO_4$ 0.2; $MnSO_4$ 0.05; Tween-80 1.0 ml/l; and agar 2.0; at a pH of 6.0 (De Angelis et al. 2002). The optical densities of the liquid cultures in MAM broth were adjusted to 1.0, and 1 % (v/v) inoculum was used in various assays. Cultures were incubated at 37 °C for 24 h and subcultured thrice in MAM broth, before being subjected to the ADI assay.

ADI activity assay

To determine enzyme activity, cultures grown for 24 h were centrifuged at 8,000 rpm for 10 min. Cell-free supernatant was assayed for extracellular protein and enzyme activity. For assaying intracellular ADI activity, cell pellets were resuspended in lysis buffer (BugBuster Protein Extraction Reagent, Novagen). Total protein was estimated by measuring absorption at 280 nm and quantified using a standard curve of bovine serum albumin. Preliminary screening of arginine-catabolizing LAB strains was based on the Nesslerization method (Imada et al. 1973). The confirmatory assay of ADI activity was based on the standard method of De Angelis et al. (2002). Briefly, under standard conditions, the reaction mixture consisted of 150 μl of 50 mM arginine, 2.3 ml of 50 mM acetate buffer (pH 5.5), 50 μl of cell wall or cytoplasm preparation, and 3.6 μl of sodium azide (final concentration, 0.05 % wt/v). Controls without substrate and without enzyme were included. After incubation at 37 °C for 1 h, the reaction was stopped by adding 0.5 ml of a solution of 2 N HCl, and precipitated protein was removed by centrifugation. The citrulline content of the supernatant was determined by the standard method of Archibald (1944). One enzyme unit was defined as the amount of enzyme required to catalyze formation of 1 μmol citrulline per min. One milliliter of supernatant was added to 1.5 ml of an acid mixture of H_3PO_4-H_2SO_4 (3:1 v/v) and 250 μl of diacetyl monoxime (1.5 % 2, 3 butanedione monoxime) in 10 % (v/v) methanol, mixed and then boiled in the dark for 30 min. After cooling for 10 min, the absorbance was measured at 460 nm. Finally, specific ADI activity was calculated as international enzyme units present per mg (IU/mg) of protein as per Kaur and Kaur (2012). Crude extract was qualitatively and quantitatively analyzed for citrulline content by high-performance liquid chromatography (HPLC) (Shimadzu, UV detector, column C-18, length-25 cm, and ID-4.6 mm), according to the method given by Bai et al. (2007).

Biochemical and molecular identification of the LAB isolate GR7

Preliminary identification of the isolate GR7 was carried out according to Bergey's Manual of Determinative Bacteriology, Ninth Edition (Holt et al. 1994). The effect of temperature

(4 °C to 55 °C), NaCl (2 % to 12 %) and pH (5.0 to 12.0) on growth of the organism was studied. Cell morphology, motility (Smibert and Krieg 1994), Gram reaction and catalase activity of the strain were studied as per standard methods. Biochemical features such as bile test, arginine dihydrolase activity, ammonia production from arginine (Niven et al. 1942), fermentation of sugars, Voges-Proskauer test, methylene red test, and indole test were studied as per standard protocols (Smibert and Krieg 1994). Molecular identification by 16S rRNA sequencing for bacterial isolate GR7 was carried out at MTCC, IMTECH, Chandigarh (India). The sequence was aligned with a non-reductant DNA database and sequence homologies were studied using the CLUSTALW program. Pairwise evolutionary distances of the homologues were calculated using Kimura's two-parameter model. A phylogenetic tree was constructed from distance matrices by the neighbor-joining method, using MEGA 5.10 software.

Genomic characterization of the *E. faecium* GR7 *arc* operon

Genomic DNA was isolated from *E. faecium* GR7using the GeNei ™ Bacterial DNA purification kit.

Amplification of arcA, arcB and arcC

The *arcA*, *arcB* and *arcC* genes were amplified from *E. faecium* GR7 by polymerase chain reaction (PCR) using the primers listed in Table 2. Primers were designed on the basis of reference sequences of *arcA*, *arcB* and *arcC* in *E. faecium* Aus 0004 (Genbank no. CP003351.1), using Gene runner 3.05 (Hastings Software Inc.), PCR primer stat and PCR product tools of Sequence manipulation suite (SMS). DNA sequences of 1,260 bp and 2,750 bp were amplified after initial denaturation of genomic DNA at 94 °C for 5 min and 32 PCR cycles (involving denaturation – 94 °C for 30 s; annealing – 58 °C for 30 s and extension – 72 °C for 3 min) using Taq DNA polymerase and a Techne programmed thermal cycler. Amplicons of 1.2 kb and 2.5 kb were further sequenced at GenScript, New Jersey, USA.

In silico analysis of sequenced ADI genes

The homology analysis of the nucleotide sequences was carried out using the National Center for Biotechnology Information (NCBI)'s $BLAST_n$ program.

Sequenced ADI operon fragments were aligned using the $BLAST_n$ tool (blast.ncbi.nlm.nih.gov). The ClustalW tool (srs.ebi.ac.uk/ClustalW) was used for sequence homology analysis. Various control regions of sequenced ADI genes, i.e., promoter, ribosomal binding site (RBS), encoded polypeptides or open reading frame (ORF) and terminators were identified based upon their homology with *E. faecium* Aus 0004 as a reference sequence using various bioinformatics tools such as $BLAST_p$, the reverse translate and translation tool of SMS, and the ORF finder tool of SMS. Stem-loops were localized with program REPEATS and their ΔG values were calculated with the program Mfold (RNA mfold, version 2.3, server [http://bioinfo.math.rpi.edu]). Fingerprints of the amino acid sequence were analysed by the P-val FingerPRINTScan tool provided with PRINTS, and used to align the Molecule Page Protein sequence to profiles derived from the PRINTS database. Multiple sequence alignment of protein was carried out using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Nucleotide sequence accession number

The sequence reported has been submitted to Genbank.

Statistical analysis of results

One-way ANOVA analysis was carried out, and results are presented as mean±standard deviation of three triplicate experiments. A probability value (p)<0.05 was used as the criterion for statistical significance.

Results

Screening of L-arginine–catabolizing LAB strains

The first step of the ADI pathway results in the production of ammonia and citrulline as an end product of arginine catabolism. Therefore, ammonia production provides a simple and rapid test to screen various bacterial isolates for their ability to degrade arginine. ADI activity was confirmed by the quantitative analysis of citrulline production. Preliminary screenings of bacterial isolates were carried out by Nesslerization. Fifty-one LAB isolates showing positive results for Nessler's test were further analysed quantitatively for citrulline production to determine ADI activity. Both extracellular and intracellular specific ADI activities of LAB isolates were determined. In this study, LAB isolate GR7 showed the highest total specific ADI activity: 0.27±0.015 IU/mg with 0.0176±0.023 IU/mg and 0.2523±0.062 IU/mg extracellular and intracellular specific ADI activity, respectively (Table 1). Citrulline production was also confirmed by HPLC analysis, where a single highest peak chromatogram detected at 190 nm with a retention time of 2.392 min confirmed the presence of citrulline in crude extract (Fig. 2). It was selected for further characterization of the strain at the biochemical and molecular level.

Table 1 ADI activity reported in lactic acid bacterial isolates

S.No	Sample	Nessler reaction	Extracellular specific ADI activity (IU/mg)	Intracellular specific ADI activity (IU/mg)	Total specific ADI activity (IU/mg)
1	MP3	+	0.0163±0.02	0.1275±0.012	0.14±0.021
2	MP4	+	0.0211±0.021	0.0804±0.012	0.10±0.01
3	MP5	+	0.0252±0.05	0.1361±0.018	0.16±0.015
4	MP6	+	0.0228±0.015	0.1001±0.016	0.12±0.015
5	MP7	+	0.0196±.061	0.2002±0.080	0.22±0.020
6	GR5	+	0.0192±.062	0.1081±0.072	0.13±0.026
7	GR6	+	0.011±0.015	0.2529±0.056	0.26±0.021
8	**GR7**	**+**	**0.0176±0.023**	**0.2523±0.062**	**0.27±0.015**
9	LP1	+	0.0159±0.005	0.1548±0.018	0.17±0.020
10	LP2	+	0.0208±0.005	0.1408±0.014	0.16±0.005
11	LP3	+	0.0171±0.030	0.2016±0.043	0.22±0.026
12	LP4	+	0.0209±0.005	0.1659±0.014	0.19±0.02
13	LP5	+	0.0175±0.020	0.2312±0.062	0.25±0.02
14	LP6	+	0.0248±0.026	0.0633±0.031	0.09±0.01
15	LP7	+	0.0139±0.015	0.1377±0.018	0.15±0.025
16	PP1	+	0.0132±0.001	0.074±0.032	0.09±0.02
17	PP2	+	0.0136±0.005	0.1038±0.043	0.12±0.01
18	PP3	+	0.0161±0.061	0.0796±0.053	0.09±0.03
19	PP4	+	0.0123±0.023	0.1643±0.060	0.18±0.015
20	PP5	+	0.0216±0.009	0.1599±0.042	0.18±0.02
21	PP6	+	0.0089±0.012	0.076±0.027	0.08±0.04
22	DP1	+	0.002±0.032	0.1143±0.067	0.12±0.20
23	DP2	+	0.0025±0.018	0.1202±0.026	0.12±0.026
24	DP3	+	0.0054±0.026	0.1375±0.053	0.14±0.02
25	DP4	+	0.0045±0.008	0.0562±0.025	0.06±0.03
26	DP5	+	0.0122±0.072	0.0232±0.018	0.04±0.021
27	DP6	+	0.0136±0.043	0.1003±0.062	0.11±0.030
28	DP8	+	0.0193±0.028	0.0616±0.057	0.08±0.030
29	DP9	+	0.0205±0.031	0.1259±0.023	0.15±0.005
30	DP10	+	0.0138±0.034	0.0929±0.072	0.11±0.015
31	CP1	+	0.0137±0.041	0.2105±0.027	0.22±0.01
32	CP2	+	0.0051±0.032	0.2035±0.039	0.21±0.01
33	CP3	+	0.0445±0.042	0.0658±0.019	0.11±0.01
34	CP4	+	0.0053±0.027	0.2074±0.027	0.21±0.005
35	CP5	+	0.0168±0.051	0.0592±0.021	0.08±0.03
36	CP6	+	0.0071±0.032	0.2334±0.054	0.24±0.015
37	CP7	+	0.0068±0.030	0.1946±0.040	0.20±0.0152
38	KC1	+	0.0868±0.023	0.0476±0.013	0.13±0.0152
39	KC2	+	0.0654±0.030	0.1152±0.021	0.18±0.011
40	KC3	+	0.0332±0.026	0.0515±0.042	0.08±0.03
41	KC4	+	0.0109±0.028	0.0012±0.021	0.01±0.005
42	KC5	+	0.0129±0.038	0.0047±0.024	0.02±0.002
43	RP6	+	0.0205±0.032	0.0799±0.062	0.10±0.01
44	RP7	+	0.0078±0.040	0.0012±0.023	0.01±0.015
45	RP8	+	0.0118±0.029	0.0085±0.045	0.02±0.02
46	RP9	+	0.0447±0.031	0.1921±0.054	0.24±0.026
47	RP10	+	0.0704±0.041	0.0451±0.017	0.12±0.02
48	RP11	+	0.0305±0.034	0.0517±0.016	0.08±0.012

Table 1 (continued)

S.No	Sample	Nessler reaction	Extracellular specific ADI activity (IU/mg)	Intracellular specific ADI activity (IU/mg)	Total specific ADI activity (IU/mg)
49	RP12	+	0.0286±0.039	0.2268±0.032	0.24±0.015
50	RP13	+	0.0237±0.027	0.0105±0.056	0.03±0.03
51	RP14	+	0.0348±0.031	0.0787±0.034	0.11±0.01

Biochemical and molecular analysis of LAB isolate GR7

Based on morphological and physico-chemical features, LAB isolate GR7 has been characterized as *Enterococcus faecium*. Colonies of the GR7 isolate were observed as off-white, circular, flat, smooth, dry, and opaque. The isolate consisted of non-motile cocci that showed positive Gram staining and negative catalase activity. It was shown to grow well at a temperature range of 25–42 °C, from pH 5–12, and in the presence of 12 % salt. The isolate showed positive results for Voges Proskauer reaction, ammonia production from arginine and negative results for methyl red, oxidase, nitrate and indole tests. Isolate GR7 showed acid production from cellobiose, melibiose, maltose, lactose, sucrose and galactose, whereas no acid production was observed for inositol, dulcitol, trehalose, adonitol, sorbitol, salicin.

The partial 16S RNA sequence (1,445 bp) of *E. faecium* GR7 showed a very close homology with *E. faecium* ATCC 19434 (99.23 %), *E. villorum* LMG 12287 (98.33 %), *E. ratti* ATCC 700914 (98.27 %), *E. mundtii* CECT 972 T (98.19 %), *E. thailandicus* FP 48–3 (98.17 %), *E. faecium* AUS0004 (98 %), *E. faecalis* NRIC 0112 (98 %), *Enterococcus* sp. LMG 12316 (97 %), *E. durans* DSM 20633 (97 %), *E. hirae* NRIC 0109 (97 %), *E. sanguinicola* BAA-781 (97 %), *E. faecium* HN-N 39 (96 %), *E. lactis* CK 1025 (96 %), *E. faecalis* V583 (95 %) and *E. lactis* CK 1114 (95 %). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 100 replicates was taken to represent the evolutionary history of the taxa analyzed. The percentage of replicates trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distance (0.001) was computed using the Kimura two-parameter method (Kimura 1980). The analysis involved 14 nucleotide sequences of known homologues of *Enterococcus* species and was conducted in MEGA 5.10 (Tamura et al. 2011), as shown in Fig. 3. On the basis of cell and colony morphology, biochemical tests, 16S rRNA gene sequence analysis and strains that clustered together, isolate GR7 was identified as *Enterococcus faecium*. The 16S rRNA sequence of isolate GR7 has been deposited into the Genbank database (vide accession no. KC179714).

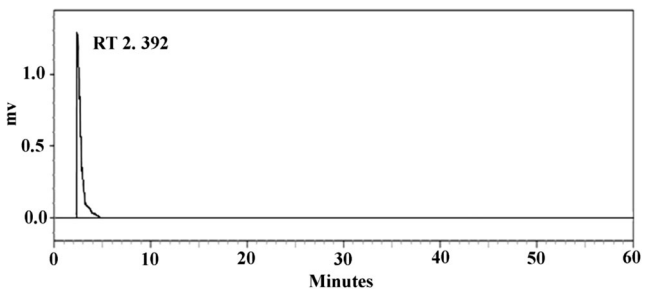

Fig. 2 High-performance liquid chromatography (HPLC) analyses of citrulline from crude extract using a C18 column and detected at 190 nm with a retention time (RT) of 2.392 min. The mobile phase was acetonitrile: 0.03 M potassium phosphate, pH 3.2 (20:80); flow rate was 0.5 ml/min; column temperature was 30 °C; and sample injection volume was 20 μl

Genetic organization and characterization of the ADI gene cluster

To obtain genetic information on the ADI operon of *E. faecium* GR7, this along with surrounding fragments encompassing the partial operon were amplified and sequenced. In order to amplify *arcA* (ADI), *arcB* (OTC) and *arcC* (CK) genes, synthetic primers were designed from conserved regions deduced from an alignment of nucleotide sequences from *E. faecium* Aus 0004 (Genbank no. CP003351.1) and *E. faecium* DO (Genbank no. CP003583.1). PCR reactions were performed on genomic DNA isolated from isolate GR7, and gave amplified products of 1,260 bp and 2,553 bp with primers designed for *arcA* and *arcBC* respectively (Table 2, Fig. 4a and b).

Nucleotide Sequence analysis of 3,906 bp (consisting of 1,260-bp-long *arcA* and 2,553-bp-long *arcBC* regions) of *E. faecium* GR7 showed 95 % and 79 % similarity with *E. faecium* Aus 0004 (Genbank no. CP003351.1) and *E. faecalis* ATCC 29212 (Genbank no. CAC41342.1), respectively. Analysis revealed the presence of three non-overlapping reading frames (ORFs) arranged contiguously in the ADI-encoding strand (Fig. 5). All of them have an upstream Shine Dalgarno box and start with an ATG codon, suggesting that they are translated. The genetic organization of the ADI operon in *E. faecium* GR7 is shown in Fig. 5. In the *arc* gene cluster, all open reading frames lie on the anti-sense strand, followed by putative hairpin terminators (Fig. 5). Sequence comparison revealed significant similarity of *E. faecium* GR7 (GenBank no. AGN03853.1) at the amino acid level to previously characterized gene products of the arc operon of *E. faecium* Aus 0004 (GenBank no. AFC63820.1). Sequence analysis depicted its 99 % identity to the *E. faecium*

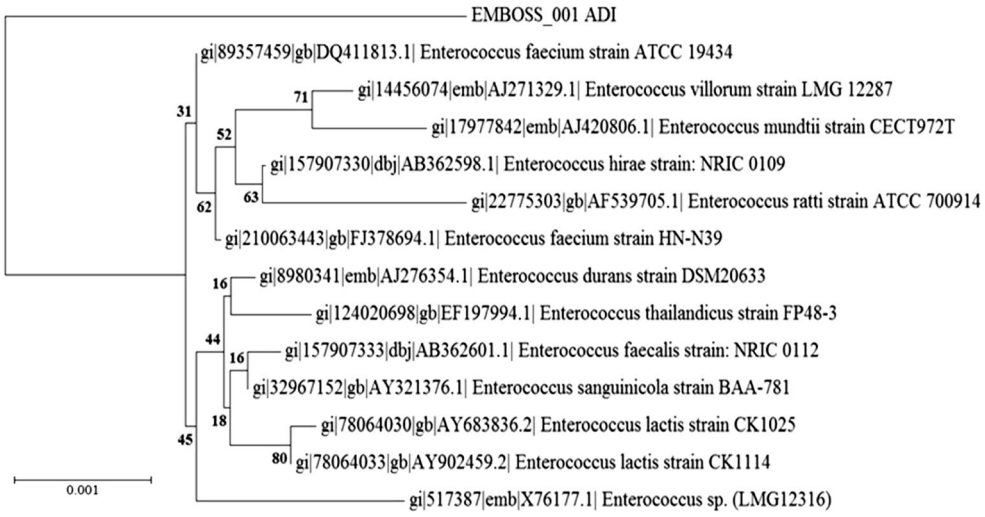

Fig. 3 Unrooted phylogenetic tree derived from 16S rRNA sequence analysis showing the relationship and evolutionary distance of *Enterococcus faecium* GR7 and its homologues. Scale bar, 0.1 % estimated divergence. Numbers indicate bootstrap values for branch points

Aus 0004 *arcC* product; 100 % to that of *arcB* and 99 % to that of *arcA* of *E. faecium* Aus 0004. According to this data, the ORFs could encode the following putative proteins: carbamate kinase (315 aa), ornithine transcarbamylase (339 aa) and arginine deiminase (409 aa). So, these genes were named as *arcC*, *arcB, arcA*, after the designations proposed for the genes of the *arc* operon of *E. faecalis* ATCC 29212. A ribosomal binding site of five nucleotides (GAGGA) is present 32 bp upstream from *arcC*, just one bp upstream from *arcB* and 11 bp upstream from *arcA*. Sequence analysis of the promoter region (Fig. 5) revealed that the promoter is situated 83 bp upstream from the ribosomal binding site of *arcA*, with the −10 (**T**A**AT**TA), TA/TATT/AA/T and −35 (**AAT**AT**T**), AA/TTA/TT/AT putative boxes separated by 17 bp downstream of the −10 region, which, however, shared bases (bold and underlined letters) with the typical consensus sequence (Blancato et al. 2008). In our study, based on partially amplified gene sequence products, the *arcCBA* operon of *E. faecium* GR7 was proposed (Fig. 5) and deposited in the Genbank database (accession no: KC700335).

Multiple sequence alignment of the deduced amino acid sequences of *arcA*, *arcB*, and *arcC* gene products of *E. faecium* GR7 (GenBank no. AGN03853.1) shows high homology with ADI genes of *E. faecium* AUS0004 (GenBank no. AFC63820.1) and *E. faecium* ATCC 29212 (GenBank no. CAC41341.1), as shown in Fig. 6. Conserved domains of the ADI pathway enzymes were evaluated using p values and the FPScan tool of PRINTS, which depicted fingerprints for ADI pathway genes of *E. faecium* GR7. Arginine deiminase is the first enzyme of the ADI pathway; it catalyses the conversion of L-arginine to L-citrulline and ammonia. A six-element fingerprint of ARGDEIMINASE, a signature for the bacterial arginine deiminase protein family, was identified in *arcA*, which showed all six conserved motifs as shown in Fig. 6. The putative protein of *arcB* showed a high degree of similarity with OTCASE, AOTCASE, and ATCA SE. The C-terminal regions of these enzymes showed very high similarity to the aspartate carbamoyltransferases, which bind to carbamoyl-phosphate (Mosqueda et al. 1990). This region contains a highly conserved Cys residue (in a His-Cys-Lys-Pro motif) implicated in ornithine binding (Huygen et al. 1987). OTCASE is a five-element fingerprint that provides a signature for ornithine carbamoyltransferases (Fig. 6). Carbamate kinase is involved in the last step of the ADI pathway, converting carbamoyl phosphate and ADP into ammonia, carbon dioxide and ATP. CARBMTKINASE is a seven-element fingerprint that provides a signature for the bacterial carbamate kinases. *E. faecium* GR7 showed all seven conserved motifs of varying lengths (Fig. 6).

Discussion

The ADI pathway has been explored in various strains of LAB belonging to the genera *Enterococcus, Lactobacillus,*

Table 2 Properties of the *arcA* and *arcBC* primers used in the study

Gene	Primer	Sequence	Length	T_m(°C)	% GC	Product size
arcA	Forward	5′-gactgccgtcaggaaacaaattacttcaag-3′	30	60	43.33	1,260 bp
	Reverse	5′- gcaacacaacatggataaacctattcacg-3′	29	59	41.38	
arcBC	Forward	5′- cgttgtatcttcttcattcggcttcc-3′	26	58	46.15	2,750 bp
	Reverse	5′- caatcgctccaggtgttgtagtcact-3′	26	60	50	

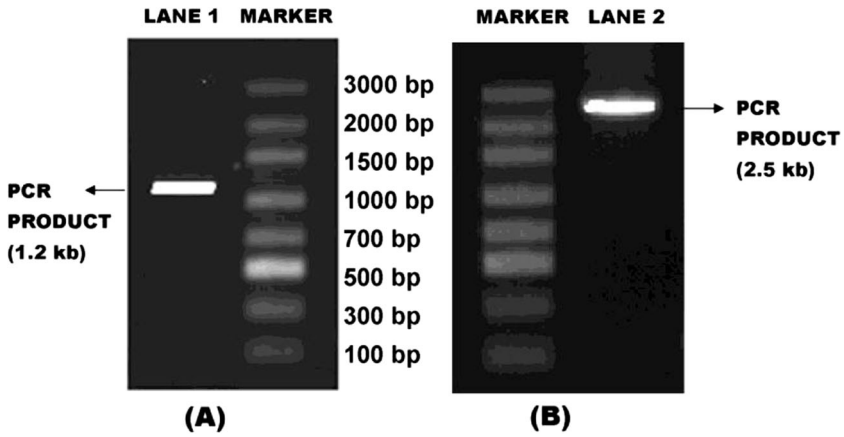

Fig. 4 (**a**): *arcA* PCR product (lane 1) and marker DL3000 (lane 2). (**b**): *arcBC* marker DL3000 (lane 1) and PCR product (lane 2)

Lactococcus, Leuconostoc, *Streptococcus* and *Weissella* (Mackey and Beck 1968; Spano et al. 2007; Vrancken et al. 2009; Kaur and Kaur 2012). The generation of ATP by arginine catabolism via the ADI pathway in bacteria plays important role in overcoming acid and oxygen stress, as well as nutrient starvation; and it supplies carbamoyl phosphate, which is essential for de novo synthesis of pyrimidines (van den Hoff et al. 1995; Champomier-Verges et al. 1999). In some bacteria, such as *Pseudomonas aeruginosa* and *Bacillus licheniformis*, ADI permits growth under anaerobic conditions (D'Hooghe et al. 1997). In others, such as oral streptococci (Dong et al. 2002) and *Lactobacillus sakei* (Zuniga et al. 1998), expression of ADI is under the control of carbon catabolite repression (CCR) and its expression increases in the presence of arginine (Dong et al. 2004). Furthermore, ADI provides protection against acidic stress by the production of ammonia in oral streptococci (Casiano-Colon and Marquis 1988).

Different LAB strains differ with respect to ADI activity, which varies according to their source of isolation and nature of the strain (Arena et al. 1999). The specific ADI activity of various LAB ranges from 0.10 to 1.62 IU/mg (Liu et al. 1996; De Angelis et al. 2002). Most LABs studied so far have conserved *arcA* (ADI), *arcB* (OTC) and *arcC* (CK) genes in the ADI operon. Additional genes such as *arcR* (regulator) and *arcD* (arginine/ornithine (A/O) antiporter), a membrane transport protein that catalyzes the stoichiometric and electroneutral exchange between extracellular arginine and intracellular ornithine, are also reported in the LAB *arc* operon (Liu et al. 2008; Poolman et al. 1987). The leakage of ADI pathway intermediates and/or end products across the membrane (i.e., release, uptake and its further intracellular conversion), is observed to be a pH-dependent phenomenon (Vrancken et al. 2009). Both extracellular and intracellular ADI activities have been reported earlier in various LAB species. Extracellular activity was reported among *Lactobacillus sakei* (Montel and Champomier 1987), *Streptococcus sanguinis*, *Streptococcus ratti* (Casiano-Colon and Marquis, 1988), and *Enterococcus faecalis* NJ402 (Li et al. 2006), whereas intracellular activity was reported among *Leuconostoc oenos* OENO and *Lactobacillus buchneri* CUC-3 (Liu et al. 1996), *Streptococcus faecalis* and *Streptococcus faecium* (Mackey and Beck, 1968). Authors used MAM medium containing Tween80 for the isolation and cultivation of the GR7 strain from a dairy product that is known to induce formation of small pores in the cytoplasmic membrane, thereby causing leakage of intracellular metabolites. Because of this, both intracellular and extracellular ADI activities have been reported in *E. faecium* GR7, and the isolate showed a very high specific ADI activity of 0.27±0.015 IU/mg.

In many bacteria, the ADI genes, namely ADI, OTC and CK, are found to be associated with additional regulatory

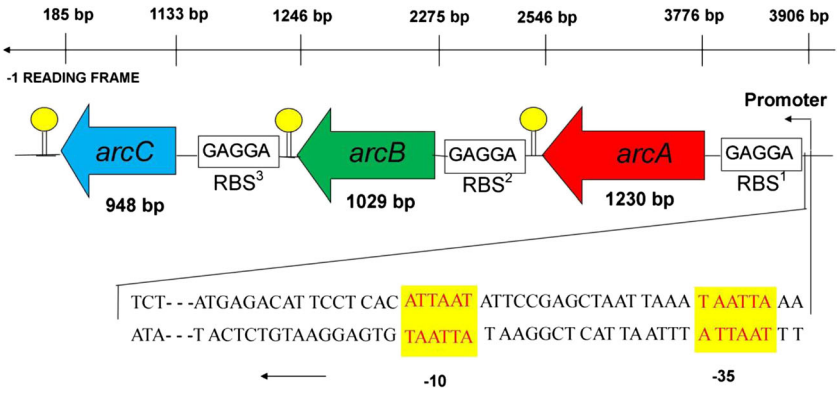

Fig. 5 Gene composition and organization of the ADI operon in *E. faecium* GR7, where RBS stands for ribosomal binding sites and lollipop symbols represent terminators

Fig. 6 Alignment of *E. faecium* GR7 *arcA*, *arcB*, and *arcC* gene products with ADI from *E. faecium* AUS0004 and *E. faecium* ATCC 29212 [Where A, B, and C represent deduced amino acid sequences of ADI from *E. faecium* GR7 (GenBank no. AGN03853.1), *E. faecium* AUS0004 (GenBank no. AFC63820.1), and *E. faecium* ATCC 29212 (GenBank no. CAC41341.1), respectively]. The boxes highlight signature ADI sequences

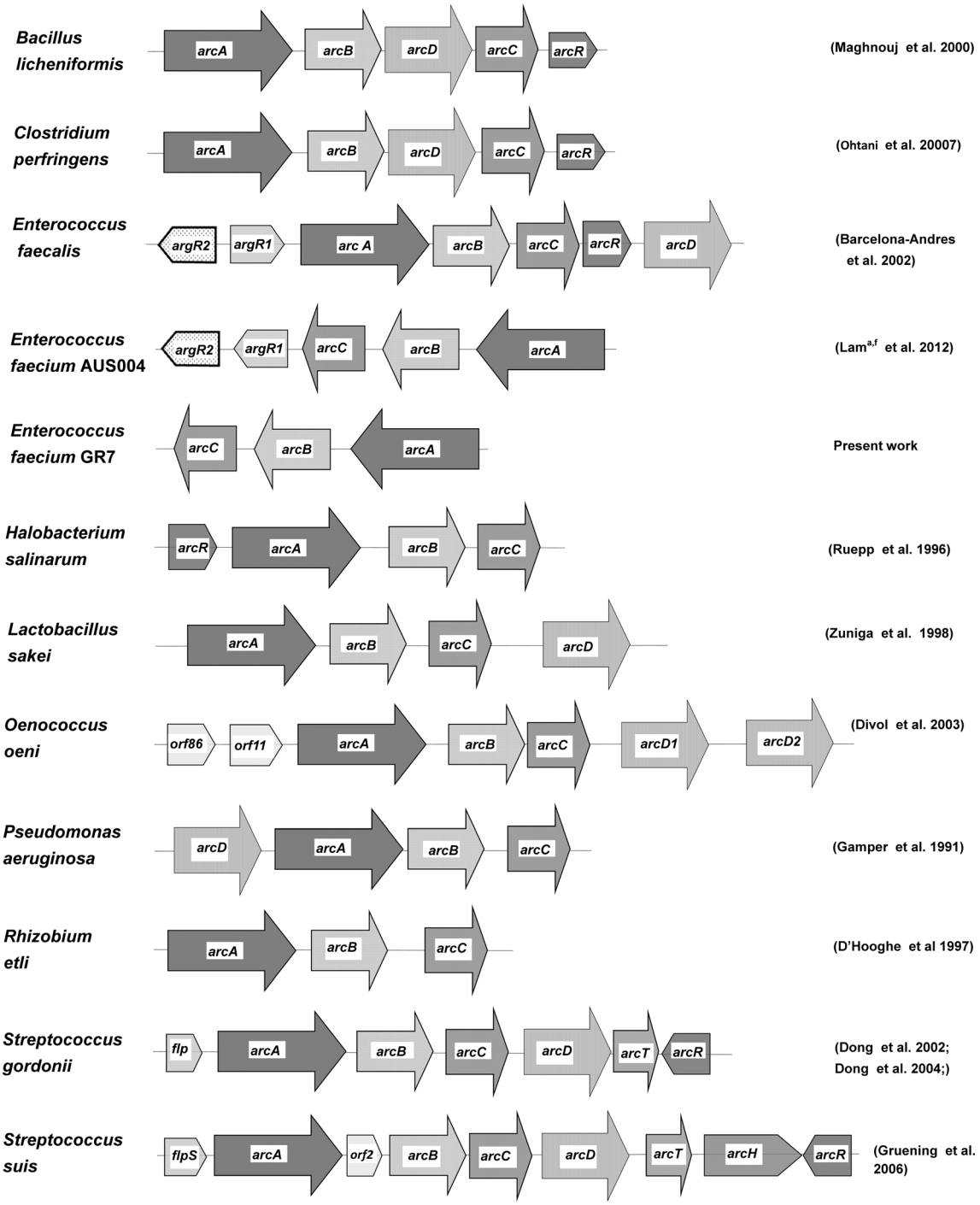

Fig. 7 ADI gene composition and genetic organization in various bacterial strains

genes belonging to different families of transcriptional regulators, not-yet-characterized genes, and genes encoding putative transport proteins (Fig. 7). The gene arrangement, regulation and biological role of ADI differs among bacterial species (Casiano-Colon and Marquis 1988; Gamper et al. 1991; D'Hooghe et al. 1997; Barcelona-Andres et al. 2002; Dong et al. 2004). Among the studied ADI pathway gene clusters, those of *E. faecium* AUS004 and *E. faecium* GR7 resemble each other the most, since they are on the anti-sense strand and appear to have the same gene order, although *E. faecium* AUS004 has the extra genes *argR2* and *argR1* (Lam et al. 2012). Genetic studies have shown that organization of the *arc* operon in LAB is very complex, due to the presence of additional and duplicated genes such as *arcH*, *arcT flp* and *orf* next to the ADI pathway gene *arcABCD* (Divol et al. 2003; Dong et al. 2004; Gruening et al. 2006); also, the order of genes varies in different species, and *arcD* organization seems to be only partially conserved among

organisms (Fig. 7). The ADI pathway is activated by the*arcR* gene and expression of the *arc* operon is inducible by arginine in *Bacillus*, *Clostridium*, *Enterococcus*, and *Halobacterium* sp. (Ruepp and Soppa 1996; Ohtani et al. 1997; Maghnouj et al. 2000; Barcelona-Andres et al. 2002).

We have investigated the arginine hydrolyzing potential of *E. faecium* GR7, which was isolated from a locally available dairy product. The genes encoding different enzymes involved in arginine catabolism were identified in *E. faecium* GR7. On the basis of 16S rRNA sequence, the nucleotide sequence of the ADI operon and amino acid sequences of the ADI enzymes, *E. faecium* GR7 was identified and its closest similarity was predicted to be with *E. faecium* AUS004. Previous studies suggest that ADIs isolated from various Gram-positive and Gram-negative bacteria could be potential therapeutic agents against arginine-auxotrophic tumors, mainly melanomas, hepatocellular carcinomas and retinoblastoma. Our future work will focus on the purification of the arginine deiminase of *E. faecium* GR7, as well as exploring its potential as an anticancer enzyme against various neoplastic arginine-auxotrophic cell lines, as an anticancer enzyme.

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Conflict of interest The authors declare no conflict of interests.

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